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With amended claims and statement.

(54) Title: QUANTITATIVE IMMUNOCYTOCHEMISTRY ASSAY

(57) Abstract

An internal control or standard is provided for the direct quantitative assay by immunocytochemistry of target molecules in tissue specimens and the like. The control is subjected to the same conditions including immunostaining as the tissue specimen. Optical density of the control and the specimen after staining is compared, preferably by a cell analysis computer system.

* See back of page

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QUANTITATIVE IMMUNOCYTOCHEMISTRY ASSAY

This application is a continuation of United States application Serial No. 07/412,450 filed 26 September 1989, which is still pending.

This invention relates to the direct quantitative determination by immunocytochemistry of target molecules in tissue specimens.

BACKGROUND OF THE INVENTION

Assays to determine the presence or absence of target molecules in tissue specimens typically entail the running of an appropriate biochemical test on a tissue homogenate. Assays for estrogen receptor (ER) and progesterone receptor (PR) are of value in predicting tumor response and are routinely done in homogenates of breast cancer.

Tissue homogenates for quantitative biochemical assays are based on a variable number of normal cells and cells such as cancer cells which express target molecules. Immunocytochemistry has a theoretical advantage over such biochemical assays in that it directly measures the presence or absence of the target molecules in the relevant cells.

To date, however, this advantage has not been realized because current immunocytochemistry is based on a visual estimation of the intensity of the immunostaining reaction product. This procedure has major drawbacks which preclude its use as a precise, reproducible, quantitative assay. Among other things:

(1) The various tissue processing steps (fixation time, type of fixative, dehydration, embedding and related procedures) are all responsible for some variation, in particular loss of antigen, which modifies the immunostain signal.

These variables are difficult to control as they depend on fixative concentration, temperature, contaminants and, importantly, time.

(2) The thickness of the tissue section is difficult to control--an important factor because the signal strength increases as a function of section thickness.

(3) The staining procedure will indicate differences in intensity of immunoreactivity depending upon multiple variables, such as antibody concentration, timing, type of chromogen used, and the like.

SUMMARY OF THE INVENTION

This invention replaces the visual estimation procedure of the prior art with a practical technique for utilizing the capability of immunocytochemistry to measure quantitatively the presence or absence of a target molecule in the cells of a tissue or other specimen. More particularly, the invention provides a control or internal standard containing a known amount of antigen (or other target molecules) to be processed concurrently with the tissue specimen. The control is thus subjected to all of the same conditions as the tissue specimen to be assayed. For example, a decrease of 30% in the availability of an antigen to be detected will equally affect the control and the specimen under examination. Because the control has cells with a known amount of antigen or other target molecules, a compensating factor can readily be determined, and a quantitative assay of the tissue specimen accomplished.

DESCRIPTION OF THE INVENTION

The control provided by this invention may be a section or slice of a medium, such as gelatin, agar or any other aqueous embedding medium, having embedded therein cells, such as MCF7 (a known breast cancer cell line which expresses ER and PR) or BT474 (a known cell line which expresses a breast cancer oncogene), expressing a defined amount of a target molecule. More specifically, cells grown in tissue culture are suspended in the gelatin or other embedding medium which is allowed to solidify by cooling or polymerization. The solidified medium containing the cell suspension is cut into sections or slices of appropriate thickness, e.g., a thickness of from about 2 to about 5 mm.

Choice of cell type depends upon the type of antigen or other molecules to be measured. There is a plethora of cells of many types expressing a variety of antigens or other molecules. Transfected cells may be used. Target molecules which may be quantified in a specimen by use of this invention include, for example, proteins expressed by oncogenes, cell growth factors, and any of the various molecules that control cell proliferation.

The pathologist receiving, for example, a breast biopsy suspected of containing cancer cells includes a control slice embodying the invention into the cassette to insure that both the tissue sample and the control are concurrently subjected to all ensuing procedures through immunostaining. The optical density of the stained cancer cells in the biopsy sample is compared to that of the cells in the control which acts as an internal standard. Preferably, the comparison is made by use of a computerized cell analysis system, such as a CAS 200 image analyzer (Cell Analysis Systems, Inc., Lombard, Illinois).

EXAMPLE

Cells grown in tissue culture (MCF7 and BT474), known to express a defined amount of the antigens to be measured, were briefly fixed in paraformaldehyde and suspended in a 3% agar solution (Difco, Detroit, Michigan) at 56°C and allowed to gel. Uniform slices, 3 mm thick, were fixed in formaldehyde for periods of time ranging from 4 to 72 hours and processed together into a single paraffin block. The cross-sectional dimensions of the block were about 2 by about 2.5 cm. Sections cut at 5 microns were immunostained for estrogen receptor (ER-ICA, Abbott, Illinois) and cERB-b2 oncoprotein (Triton Bios., Alameda, California). The intensity of the immunoreactivity was measured for each quantitation of immunocytochemistry gel with a CAS 200 image analyzer. Progressive reduction in the intensity of the immunoreactivity, which correlated with the lengthening of the fixation time was detected with both antigens. Significantly, such reduction was not noticeable by conventional microscopy in the ER-ICA stains.

WHAT IS CLAIMED IS:

1. A control for use in quantitative immunocytochemistry assays which comprises a section of a solidified embedding medium having suspended therein cells which express a defined amount of a preselected target molecule.

2. A control as defined by claim 1 in which the target molecule is an antigen, a protein expressed by an oncogene, a cell growth factor, a receptor molecule, or a molecule that controls the proliferation of a cell.

3. A control as defined by claim 1 or claim 2 in which the preselected target molecule expressed by said cells embedded in said solidified embedding medium is an antigen.

4. A control as defined by claim 1 or claim 2 in which said antigen is an estrogen receptor or a progesterone receptor.

5. A control for use in the quantitative immunocytochemistry assay of a breast tissue for estrogen and progesterone receptors which comprises a section of solidified agar having embedded therein MCF7 cells which express a defined amount of estrogen receptors and progesterone receptors.

6. An immunocytochemical method for the direct, quantitative determination of the presence or absence of a target molecule in the cells of a tissue specimen which comprises concurrently subjecting said specimen and a control as defined by claim 1 to all of the same processing steps including immunostaining and thereafter comparing the optical density of the stained tissue specimen cells with the optical density of the control cells.

7. A method as defined by claim 6 in which the comparison of the optical density of the stained tissue specimen cells with the optical density of the stained control cells is made by use of a computerized cell analysis system.

8. A method as defined by claim 6 or claim 7 in which said target molecule is an antigen.

AMENDED CLAIMS

[received by the International Bureau on 14 March 1991 (14.03.91) original claims 1-8 cancelled; new claims 9-16 added (3 pages)]

9. A quantitative immunocytochemical assay which comprises:

(i) suspending cells expressing a known, defined amount of a target molecule in an aqueous agar or gelatin gel forming medium;

(ii) causing said suspension to form an agar or gelatin gel having said cells suspended therein;

(iii) determining the optical density of a section of said gel;

(iv) placing said section of said gel in a tissue cassette together with a specimen of the tissue to be analyzed for said target molecule, such that said section and said tissue specimen are concurrently fixed, processed and embedded;

(v) determining the optical density of said fixed processed and embedded section and tissue specimen;

(vi) measuring the difference between the optical density of the section as determined in step (iii) with the optical density of the section as determined in step (v); and

(vii) utilizing the optical density difference measured in step (vi) to provide a quantitative immunohistochemical determination of the target molecule content of said tissue specimen.

10. An assay as defined by claim 9 in which said target molecule is an estrogen receptor or a progesterone receptor.

11. A quantitative immunocytochemical assay for a target molecule which comprises:

(i) concurrently fixing, processing and embedding

a section of an agar or gelatin gel containing cells expressing a known amount of a target molecule and

a tissue specimen to be assayed for expression of said target molecule;

(ii) quantitatively determining the difference in the target molecule content of said section of said gel before and after fixing, processing and embedding;

(iii) quantitatively determining the target molecule content of said tissue specimen after said concurrent fixing, processing and embedding; and

(iv) utilizing the difference in the target molecule content of said section as determined in step (ii) to provide a quantitative assay of the target molecule content of said tissue specimen to be assayed.

12. An assay as defined by claim 11 in which the difference in target molecule content of said section determined in step (ii) is determined by optical density measurement of said section.

13. An assay as defined by claim 11 or 12 in which said target molecule is an estrogen receptor or a progesterone receptor.

14. A section of an agar or a gelatin gel having suspended therein cells expressing a known, defined amount of a target molecule.

15. A section as defined by claim 14 in which said target molecule is a protein expressed by an oncogene, a growth factor, or a receptor.

16. A section as defined by claim 14 in which said cells are MCF7 cells or BT474 cells.

STATEMENT UNDER ARTICLE 19

Receipt of the International Search Report dated 25 January 1991 is acknowledged.

Attention is respectfully invited to the attached paper by applicant entitled "Immunohistochemistry vs. Molecular Biology: Which Tool When for Diagnostic Pathology". This paper is to be published in 1991 in

Molecular Pathology. The whole of the paper is instructive to place the claimed invention in context with the prior art. Please note particularly the text beginning with the heading "Quantitative Immunohistochemistry" in the left column of page 19. The invention is described therein.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05429

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/569, 33/574

U.S.Cl.: 424/3,7.1; 435/7.25; 436/8,10,813

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [4]	Classification Symbols
U.S.	424/3,7.1; 435/7.25; 436/8,9,10,11,12,13,14,15,16,17,18,19,64, 813

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US, A, 4,816,410 (Healy, Jr. et al.) 28 March 1989, see the entire document.	1-3,6,8 4-5,7-8
Y	Wick et al., Immunofluorescence Technology -Selected Theoretical and Clinical Aspects, Published 1982 by Elsevier Biomedical Press, Amsterdam (N.Y.), see pages 86-90.	7-8

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
11 December 1990	25 JAN 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Robert J. Hill. ROBERT J. HILL, JR.